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(54) Title: METHOD FOR IDENTIFYING TRANSGENIC PREIMPLANTATION EMBRYOS

(57) Abstract

Methods for identifying a transgenic preimplantation embryo comprising analyzing cells obtained from an embryo into which foreign DNA has been delivered using in situ hybridization analysis are disclosed as well as improved methods for making transgenic animals.

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METHOD FOR IDENTIFYING TRANSGENIC PREIMPLANTATION EMBRYOS

Background of the Invention

A "transgenic" animal is one whose DNA (i.e. heriditary material) has been augmented with DNA obtained from a source other than parental germplasm. Usually the source of the exogenous DNA is a different animal or a human. A variety of nucleic acid transfer techniques, including microinjection, cell fusion, electroporation, retroviral transformation, and chemical precipitation have been used to insert genes from one animal into another and thereby create a transgenic animal. Of the currently available techniques, microinjection is probably the most commonly used for introduction of foreign DNA into embryos.

In microinjection, highly purified copies of a specific gene are injected into a fertilized egg. The egg is then either cultured in vitro or surgically implanted into a female's reproductive tract. Currently, microinjected embryos are cultured or implanted without regard to their confirmation as transgenics. However, since only about 10-25% of resultant liveborn offspring will be bonafide transgenics, this process is very inefficient.

The polymerase chain reaction (PCR) technique, which allows the amplification of specific short regions of DNA to levels that can be detected by DNA blotting, has been used to detect the presence of a transgene in a preimplantation mouse embryo (King, D. and R.J. Wall Molec. Reprod. Dev. 1: 57-62 (1988); Nenomiya, T., et. al., Molec. Reprod. Dev. 1:242-248 (1989)). Although PCR can be useful for detecting the presence of a certain DNA sequence in a cell, the technique does not provide any information on whether a sequence is integrated into a chromosome or if integrated, the number or location of integration sites. Therefore, using PCR, one could not distinguish, for example, between a) 30 copies of a transgene integrated at

a single site, b) 15 copies of a transgene, each integrated at two sites or c) 10 copies of a transgene integrated at a single site and 20 copies nonintegrated and floating around in the nucleus. Further, single cell PCR is nontrivial due to the limited amount of genetic material available for analysis. Single cell PCR for only one single copy transgene is even less trivial, particularly due to problems with contamination.

A method for analyzing transgenic preimplantation embryos to determine whether foreign DNA has integrated into the host chromosome and to determine the number and location of integration sites is needed.

Summary of the Invention

In general, the invention features a method for identifying a transgenic preimplantation embryo using in situ hybridization analysis. In one embodiment, the invention features the steps of: a) obtaining an embryo; b) delivering foreign DNA into the embryo to obtain an embryo containing foreign DNA; c) culturing the embryo containing foreign DNA; d)biopsying the embryo thereby obtaining an embryonic cell; and e) analyzing the embryonic cell by in situ hybridization using a probe which is capable of hybridizing with the foreign DNA, wherein a positive hybridization signal indicates that the biopsied cell was obtained from a transgenic embryo.

In another embodiment, the invention features a method for identifying a transgenic embryo, which is the result of mating a first transgenic "founder" animal with a second animal of the same species, but of a sex opposite the sex of the first transgenic animal. This second embodiment comprises: a) obtaining an embryo which is the result of mating a transgenic "founder" animal with an animal of the same species, but of a sex opposite to the sex of the transgenic animal; b) culturing the embryo; c) biopsying the embryo thereby obtaining an embryonic cell; and d) analyzing the embryonic cell by in situ hybridization using a probe which is complementary to the foreign DNA comprising the transgene, wherein a positive hybridization signal indicates that the biopsied cell was obtained from a transgenic embryo.

In yet another embodiment, the invention features a method for identifying homozygous transgenic embryos resulting from the mating of two transgenic founder animals. In this embodiment, a transgenic heterozygous male is mated with a heterozygous female from the same line to produce an embryo. The resultant embryo is then obtained from the female, cultured and biopsied to obtain embryonic cells, which are then analyzed by in situ hybridization using a probe which is capable of hybridizing with the foreign DNA comprising the transgene, wherein the detection of two hybridization signals indicates that the biopsied cell was obtained from a homozygous transgenic embryo.

Use of the methods disclosed herein provides a means of ensuring that only transgenic embryos are implanted in a female's reproductive tract. Therefore, the disclosed methods provide an important advance over current practices wherein only about 10-25% of implanted embryos are bonafide transgenics. In addition, the subject methods provide information on the number of sites of integration, their location, the relative copy number of the foreign DNA comprising the transgene and whether a transgenic embryo is homo- or hetero- zygous.

Detailed Description of the Invention

The subject invention relates to the finding that in situ hybridization techniques can be used to detect the presence of a foreign gene which has integrated into a host chromosome. Based on this finding, transgenic embryos, (including embryos obtained from animals, fish, amphibians, insects, etc.) can now be analyzed to identify bonafide transgenics. Embryos which are confirmed as being transgenic can then be cultured in vitro or implanted in a female's reproductive tract and carried to term to produce a transgenic animal. The following methods are described with reference to animal embryos. However, it is expected that the disclosed methods can be carried out on other embryos (e.g.fish, amphibian, insect, etc.) without necessitating undue experimentation.

One method of the subject invention involves analyzing a transgenic embryo which results from the delivery of foreign DNA into an embryo. As used herein, "foreign DNA" refers to genetic material obtained from a source other than parental

germplasm. Methods for acquiring embryos and delivering foreign DNA into the embryos (e.g. via microinjection, cell fusion, electroporation, chemical precipitation (e.g. calcium phosphate) and retroviral transformation) are well-known in the art (For techniques specific for animals, See e.g. Manipulating the Mouse Embryo: A Laboratory Manual, Hogan, Constantini and Lacy eds. (Cold Spring Harbor Laboratory (1986)). Preferably DNA is delivered into an embryo at a very early stage in development so that only a small frequency are mosaic (i.e. an embryo in which integration of the foreign DNA occurs after the one cell stage). Because mosaic embryos do not contain integrated DNA in every cell, analysis of a biopsied cell obtained from a mosaic transgenic animal may not be representative of the resultant animal.

Alternatively, a transgenic embryo can result from mating a first known transgenic with a second animal of the same species but of a sex opposite to the sex of the first known transgenic animal. The second animal can be either transgenic or non-transgenic. Methods for mating animals and obtaining transgenic embryos are also well known. (See e.g. Manipulating the Mouse Embryo: A Laboratory Manual, Hogan, Constantini and Lacy eds. (Cold Spring Harbor Laboratory (1986)).

Once obtained, transgenic embryos can be cultured in embryo culture media, such as CZB media (Chatot, C.L., et. al. J. Reprod. Fertil. 86:679-688 (1989); Chatot, C.L. et. al., Biol. Reprod. 42:432-440 (1990)). When cultured cells reach an appropriate cell stage which may differ according to the species, they can be biopsied to obtain one or more cells for in situ hybridization analysis. The biopsy procedure involves making a hole in the coating surrounding an embryo either by mechanical slitting or by chemical treatment in an embryo biopsy medium (Gordon, J.W. and I. Gang, Biol. of Reprod. 42:869-876 (1990)). One or more cells is then collected, e.g. using a micropipet.

Biopsied cells can then be analyzed, while the remainder of the embryo can be returned to standard embryo culture medium. Methods for performing in situ hybridization are well-known in the art. In general, a cell sample is deposited or placed onto a slide, rendered available for hybridization, contacted with a probe and allowed to hybridize. Detection of hybridization is indicative of the presence in the cell

of a sequence which is complementary to the probe. Fluorescent in situ analysis is preferred for use in the subject invention, because it can be accomplished faster (within 24 hrs), multiple target sequences can be detected at one time and hybridization signals can be more precisely localized. Following analysis of biopsied cells, embryos which have been identified as transgenics can be cultured in vitro or transferred to the uteri of pseudopregnant recipients (for methods of implanting animal embryos See e.g. Manipulating the Mouse Embryo: A Laboratory Manual, Hogan, Constantini and Lacy eds, (Cold Spring Harbor Laboratory (1986)).

Probes for analyzing transgenic embryos can be obtained commercially. In addition, probes labelled with detectable marker can be prepared from nucleic acid molecules according to well-known procedures. Such techniques include incorporation of radioactive labels, direct attachment of fluorophores or enzymes, and various chemical modifications of the nucleic acid fragments that render them detectable immunochemically or by other affinity reactions. A preferred method of labelling is by nick translation using a haptenated nucleoside triphosphate (e.g. biotin labelled dUTP) or by random primer extension (Feinberg & Vogelstein, Anal. Biochem. 137:266-267 (1984) (e.g. multiprime DNA labeling system (Amersham) substituting dTTP with Bio-11-dUTP (Langer, P.R., et. al., Proc. Natl. Acad. Sci. USA, 78:6633-37 (1981): Brigati, D.J., et. al., Virology, 126:32-50 (1983)).

An appropriate probe for use in identifying a transgenic embryo consists of a nucleic acid sequence which is complementary to the foreign DNA comprising the transgene, so that hybridization and in situ detection of the hybridized probe on the host chromosome indicates that the foreign DNA has integrated. Host chromosome specific probes can be used in conjunction with a foreign DNA specific probe to act as a positive control for determining hybridization efficiency. In addition, a host chromosome specific probe can be used as a fiduciary marker to help localize the integration site of the foreign DNA.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

Example 1: In Situ Analysis of Preimplantation Embryos Which Have Been Microinjected With a Transgene

Embryo Retrieval and Microinjection

Egg masses containing 1-cell mouse embryos were isolated from excised oviducts of superovulated CD1 females (Charles River Labs, Wilmington, MA) that had been mated overnight with B6D2F_1 males (C57B1/6 x DBA/2 F_1 hybrid males from Taconic (Germantown, N.Y.)). Cumulus cells were removed by a brief exposure to hyaluronidase. Embryos were transferred through three drops of M2 medium (Manipulating the Mouse Embryo: A Laboratory Manual ed. Hogan, Constantini and Lacy (Cold Spring Harbor Laboratory, 1986), placed into culture in CZB medium containing glucose and Bovine Serum Albumin (BSA) and the cultures were incubated at 37C in 5% CO_2 in air. Pronuclear stage 1-cell embryos in a holding drop of M2 medium were microinjected with 1 ng/ul of an appropriate construct (Bc30, i.e. the goat beta casein promoter driving the human alpha-1-antitrypsin gene, which is about 15kb in size). Injections were almost exclusively into the larger male pronucleus. The injected embryos were then either processed for fluorescent in situ hybridization (FISH) or cultured in CZB medium containing glucose and BSA for 48-65 hrs (to the 8-16 cell morula stage) at 37C in an atmosphere of 5% O_2/5% CO_2/90% N2 prior to processing for FISH.

Cell Preparation for FISH

The zona pellucida was removed from the injected eggs and morula by a brief exposure to prewarmed acidic Tyrodes solution (Manipulating the Mouse Embryo: A Laboratory Manual ed. Hogan, Constantini and Lacy (Cold Spring Harbor Laboratory, 1986). The morula stage embryos were then incubated in calcium-free CZB medium with 5mg/ml BSA for 20 min. at 37C in 5% CO_2 in air to decompact the embryos. The embryos were then disaggregated using a flame-polishing micropipet to single/pairs of cells either individually or in groups depending on the experiment. The dissociated morula cells and the injected eggs were processed identically for FISH.

The cells were transferred into 10ul of BT buffer (T Buffer: 1mM Tris; 25mM KCl; 0.9mM $CaCl_2$ and 0.9mM $MgCl_2$ at pH 7.6; BT Buffer: T Buffer plus 20mM sodium butyrate; or BT/PMSF Buffer: BT Buffer containing a 1/100 dilution of 50mM PMSF in isopropanol on a siliconized Teflon^{Tm} masked glass slide (Cel-line Assoc, Newfield, N.J.) for 15 min. Then 20 ul of 0.3% hypotonic sodium chloride was added to the drop for 15 min. Fixation was achieved by adding 20 ul of 50% Carnoy's solution (3:1 methanol:acetic acid) in 0.3% sodium chloride or 5 ul of Carnoy's solution followed 10 min later by 40-60 ul of Carnoy's. The samples were allowed to air dry or in some cases, the excess fixative was removed prior to air drying by tipping the slide briefly on its side.

In Situ Hybridization Analysis

Fixed slides were placed on a 60 C slide warmer for 1-2 hours before hybridization. Two color fluorescence in situ hybridization experiments were performed to determine hybridization efficiency simultaneous with, but independent of, transgene identification. The transgene (BC30) and a mouse genomic probe (cBAM4, cBAM11, or cBAM14) were labeled with biotin or digoxigenin by nick translation. Each hybridization reaction contained 5-10 ng/uL of a biotin labeled mouse genomic probe, and 100 ng/uL of mouse Cot-1 DNA and 900 ng/uL salmon DNA in a cocktail of 50% deionized formamide, 6XSSC, and 10% Dextran Sulphate. A 5-10uL aliquot of hybridization cocktail was applied to each site, coverslipped, and sealed with rubber cement. Probe and target were simultaneously denatured for 7-10 min at 80C. After overnight hybridization at 37C, slides were processed in the following manner: Slides were washed for 3 X 5 min in 50% formamide/2XSSC at 42C, 1-5 min in 2XSSC at room temperature and 3-5 min in 0.1XSSC at 60C. Non-specific binding sites were blocked by incubation at 37C in 3% BSA/4XSSC for 5 min. Hybridized probe was detected with a solution containing 0.5 ng/uL FITC conjugated to antidigoxigenin and 2.0 ng/uL of Cy3 conjugated to streptavidin in 1% BSA, 0.1% Tween 20 and 4 X SSC; preparations were incubated in this solution for 20 min at 37C. Following detection, slides were washed for 3 X 5 min in 4 X SSC with 0.1% Tween 20 at 42C, then washed in 2 X SSC for at least 5 min at room temperature. Preparations were counterstained with

4',6-diamidino-2-phenylindole (DAPI) and mounted in 2.33% DABCO (Sigma, #D2522) in 100mM Tris (pH 8.0) and 90% (vol:vol) glycerol. Post hybridization washing and detection are essentially as described in Klinger et. al. Am. J. Hum. Genet. 51(1) 1992).

Results

The goal in the experiment described above was to determine the pattern of hybridization of the foreign DNA (BC30) specific probe. Specifically, the experiment was designed to determine whether there were identifiable differences between integrated and non-integrated material. Immediately after injection, about 62.5% of hybridized pronuclei displayed numerous hybridization signals. This result is what would be expected, because immediately after injection, most of the foreign DNA is not integrated. In contrast, at the morula stage, less than 4% of hybridized cells displayed multiple hybridization signals. Again, this is expected, because at this later developmental stage, most of the non-integrated foreign DNA is lost.

As shown in Table 1, the efficiency of cell recovery using the above described procedure, is significant.

TABLE 1 Efficiency of Hybridization Carried Out on Pronuclei and Morula Stage Embryonic Cells

PRONUCLEI

	Recovery	Did Not Hybridize 1-3	1-3 Signals	Diffuse or Numerous
Exp. 1	19 of 19	11	6	2
Exp. 2A	7 of 14 (50%)	4	2	1
B	14 of 14 (100%)	7	2	5
Exp. 3	18 of 20 (90%)	4	2	12

MORULA

	Recovery	Not Analyzable	Transgene Signal	No Transgene Signal
Exp. 1	57 of 70 (81%)	10	22 /or 2 discrete signals	25
Exp. 2	43 of 89 (4*%)	2	19 /or 2 discrete signals-17 multiple signals-2	21 21
Exp. 3A	34 or 44	0	10	14
	20 of 22 (91%)	0	2	9
			— 53	

Example 2: In Situ Analysis of Preimplantation Embryos Obtained From A Nontransgenic Female Mated With a Transgenic Founder Male

Embryo Retrieval and Cell Preparation for FISH

A superovulated non-transgenic CD1 female was mated with a transgenic founder male (carrying the Bc30 transgene). At 60 hr post hormone injection, embryos were flushed from the excised oviducts/uteri with Hanks Buffered Salt Solution (HBSS) and BSA. The zona pellucidae were removed from the morula stage embryos by a brief exposure to prewarmed acidic Tyrodes solution. The morula stage embryos were then incubated in calcium-free CZB medium with 5 mg/ml BSA for 20 min at 37C in 5% CO_2 in air to decompact the embryos. The embryos were then disaggregated using a flame-polished micropipet to single/pairs of cells either individually or in groups depending upon the experiment. The dissociated morula cells were transferred into 10ul of CB-PMSF on a siliconized Teflon masked glass slide for 15 min. Then 20ul of 0.3%

hypotonic sodium chloride was added to the drop for 15 min. Fixation was achieved by the addition of 20 ul of 50% Carnoy's solution (3:1 methanol: acetic acid) in 0.3% sodium chloride or 5ul of Carnoy's solution followed 10 min later by 40-60 ul of Carnoy's solution followed 10 min later by 40-60 ul of Carnoy's. The samples were allowed to air dry from Carnoy's. In some cases, the excess Carnoy's was removed prior to air drying by tipping the slide briefly on its side. In situ analysis was then performed essentially as described in Example 1.

Results

In the above-described experiment, the goal was to demonstrate good recovery and to determine the frequency of transgenic embryos as detected in embryonic cells obtained during the morula stage of embryo development. As shown below in Table 2, the efficiency of recovery is greater than 80%.

Table 2: BC30 Embryos from Mated Pairs

A	RECOVERY	TRANSGENIC	NON-TRANSGENIC	NON-HYBRIDIZE
1.	55 of 78 (71%)	26	29	0
2.	57 of 77 (74%)	30	27	0
3.	54 of 54 (100%)	27-32 (some counted 2X)		0
4A	23 of 28 (82%)	--	--	23
B	19 of 19 (100%)	--	--	19
5A	24 of 25 (96%)	0	23	1
B	15 of 17 (88%)	--	--	15

6.	38 of 40 (95%)	--	--	38
	285 of 338 (84.3%)			

NOTE:

1. Better than 80% recovery on average
2. Expected frequency of about 50% transgenic (except 5A)
3. Transgenic signal in all of these was 1-2 small discrete spots.

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

We Claim:

1. An improved method for making a transgenic animal comprising the steps of:

a) obtaining an embryo;

b) delivering foreign DNA into the embryo to obtain an embryo containing foreign DNA;

c) culturing the embryo containing foreign DNA;

d) biopsying the embryo thereby obtaining an embryonic cell and reculturing the embryo;

e) analyzing the embryonic cell by in situ hybridization using a probe which specifically hybridizes with the foreign DNA the occurrence of hybridization indicating that the embryo from which it was biopsied is a transgenic embryo

f) implanting the transgenic embryo to the uterus of a pseudo pregnant recipient; and

g) allowing the pregnancy to proceed to term thereby obtaining a transgenic animal.

2. A method of claim 1 wherein the animal is a mammal.

3. An improved method for making a transgenic animal comprising the steps of:

a) obtaining an embryo containing foreign DNA which is the result of mating a first founder animal with a second animal of the same species, but of a sex opposite the sex of the first transgenic animal;

b) culturing the transgenic embryo;

c) biopsying the transgenic embryo thereby obtaining an embryonic cell and reculturing the embryo;

d) analyzing the embryonic cell by in situ hybridization using a probe which specifically hybridizes with the foreign DNA, the occurrence of hybridization indicating that the embryo from which it was biopsied is a transgenic embryo;

e) implanting the transgenic embryo to the uterus of a pseudo pregnant recipient; and

f) allowing the pregnancy to proceed to term thereby obtaining a transgenic animal.

4. A method for determining the presence, location and relative copy number of a foreign DNA, which has integrated into a chromosome of a host embryo comprising the steps of:

a) biopsying the embryo, thereby obtaining an embryonic cell;

b) analyzing the embryonic cell by in situ hybridization using a probe which specifically hybridizes with the foreign DNA, the occurrence, location and number of hybridizations in the embryonic cell being indicative of the presence, location and relative copy number of a foreign DNA in the chromosome of the host embryo.

5. The method of Claim 4, wherein the embryo is a morula stage embryo.

6. A method for identifying a transgenic embryo which is homozygous for a foreign DNA of interest said embryo resulting from the mating of two transgenic founder animals comprising the steps of:

a) obtaining an embryo from a pregnant female that is heterozygous for a foreign DNA of interest and has been mated with a male that is heterozygous for the foreign DNA of interest;

b) biopsying the embryo, thereby obtaining an embryonic cell;

c) analyzing the embryonic cell by in situ hybridization using a probe which specifically hybridizes with the foreign DNA of interest, the occurrence of two hybridization signals on separate chromosomes indicating that the embryo from which it was biopsied is a homozygous transgenic embryo.

7. The method of Claim 6, wherein the embryo is a morula stage embryo.

8. A method for determining the presence location and relative copy number of a foreign DNA, which has integrated into a chromosome of a host embryo comprising the steps of:

a) biopsying the embryo, thereby obtaining an embryonic cell;

b) depositing the embryonic cell onto a slide;

c) contacting the cell on the slide with a solution that effects swelling of the nucleus, but not breakage of the nuclear membrane thereby obtaining swollen cells;

d) contacting the swollen cells with a hypotonic solution; and

e) analyzing the embryonic cell by in situ hybridization using a probe which specifically hybridizes with the foreign DNA, the occurrence, location and number of hybridizations in the embryonic cell being indicative of the presence, location and relative copy number of a foreign DNA in the chromosome of the host embryo.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/04072

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/00; A01K67/027; C12N15/15

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	THERIOGENOLOGY vol. 39, no. 1, 1993, page 258 LEWIS-WILLIAMS, J. ET AL. 'Selection of transgenic preimplantation mouse embryos using fluorescence in situ hybridization' see abstract	1-8
O,P, X	& Annual Conference of the Embryo Transfer Society. BATON ROUGE, LOUISIANA, USA 10-12 December 1993 ---	1-8
P,X	WO,A,9 222 647 (GENPHARM INTERNATIONAL, INC.) 23 December 1992 see page 33, line 11, paragraph 6 - page 45, line 26; claims ---	1-7
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[o] Special categories of cited documents :[10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05 AUGUST 1993	24 -08- 1993

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	WO,A,9 222 670 (GENPHARM INTERNATIONAL, INC.) 23 December 1992 see the whole document	1-7
Y	WO,A,9 200 375 (IMPERIAL CANCER RESEARCH TECHNOLOGY) 9 January 1992 see claims 21-23,27,33,34,38	1-7
Y	FIBRINOLYSIS vol. 4, no. SUP3, 4 August 1990, page 20 KRISTENSEN, P. ET AL. 'In situ hybridization analysis of urokinase-type plasminogen activator inhibitor (type 1) in the implanting mouse embryo and during wound healing' * Abstract 50 *	1-3
O,Y	& Tenth International Congress on Fibrinolysis : Indianapolis, Indiana, USA August 4-8 1990	1-3
Y	MOLECULAR BIOLOGY & MEDICINE vol. 6, no. 6, December 1989, pages 493 - 500 JENSEN, N.A. ET AL. 'Neurological disorder in transgenic mice that express the Large T antigen of Polyoma virus in the nervous system' see page 496; figures 3,4	1-3
Y	CHEMICAL ABSTRACTS, vol. 111, 1989, Columbus, Ohio, US; abstract no. 210082q, NINOMYIA, T. ET AL. 'Selection of mouse preimplantation embryos carrying exogenous DNA by polymerase chain reaction' page 167 ;column 1 ; see abstract & MOL. REPROD. DEV. vol. 1, no. 4, 1989, page 242-8	1-7

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	CHEMICAL ABSTRACTS, vol. 113, 1990, Columbus, Ohio, US; abstract no. 185758, KING, D. ET AL. 'Analysing embryos by the polymerase chain reaction' page 179 ;column 2 ; see abstract & UCLA SYMP. MOL. CELL. BIOL. NEW SERIES vol. 116, 1990, pages 33 - 45 ---	1-7
Y	CHEMICAL ABSTRACTS, vol. 110, 1989, Columbus, Ohio, US; abstract no. 167503t, KING, D. ET AL. 'Identification of specific gene sequences in preimplantation embryos by genomic amplification : detection of a transgene' page 232 ;column 2 ; see abstract & MOL. REPROD. DEV. vol. 1, no. 1, 1988, pages 57 - 62 -----	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9304072
SA 74253

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9222647	23-12-92	None		
WO-A-9222670	23-12-92	None		
WO-A-9200375	09-01-92	AU-A-	8093191	23-01-92
		EP-A-	0536213	14-04-93

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